AVIAN DISEASES 50:483–488, 2006

The Effect on Pathogenesis of Newcastle Disease Virus LaSota Strain from a Mutation of the Fusion Cleavage Site to a Virulent Sequence

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Received 7 February 2006; Accepted 3 June 2006

SUMMARY. The principal molecular determinant of virulence of Newcastle disease virus (NDV) is the amino acid sequence at the fusion cleavage activation site. To extend the understanding of the role of the fusion cleavage activation site in NDV virulence, the pathogenesis in chickens of a lentogenic LaSota isolate and two infectious clones, NDFL and NDFLtag, were compared. NDFL is an infectious clone of a lentogenic NDV strain (LaSota E13-1), and NDFLtag is the infectious clone with the fusion cleavage site sequence mutated to the virulent motif. NDFL and NDFLtag were described by Peeters *et al.* The viruses were inoculated intraconjunctivally into groups of 4-wk-old white leghorn chickens and compared in a pathogenesis study for determination of disease causation (clinical signs of disease, gross lesions, histology, virus isolation, and serology) and viral distribution (presence of viral nucleoprotein and mRNA was detected by immunohistochemistry and *in situ* hybridization, respectively). The modification of the fusion cleavage activation site to the virulent motif in the infectious clone only slightly increased disease severity and viral distribution in the pathogenesis assessment, even though dramatically increased pathogenicity of NDFLtag was confirmed by standard pathogenicity index tests. The result, that the mutated fusion cleavage site of NDV–NDFLtag had only a small influence on pathogenesis in chickens compared to either E13-1 or NDFL, suggests that the pathogenic effects of NDV are not dependent on the fusion cleavage site alone.

RESUMEN. Efecto en la patogenia de la cepa LaSota del virus de la enfermedad de Newcastle con una mutación en el sitio de corte de la proteína de fusión hacia una secuencia virulenta.

La secuencia de aminoácidos en el sitio de corte de la proteína de fusión es el principal determinante molecular de virulencia del virus de la enfermedad de Newcastle. Con la finalidad de ampliar el conocimiento sobre el papel del sitio de corte de la proteína de fusión en la virulencia del virus de la enfermedad de Newcastle, se comparó la patogenia de un aislamiento lentogénico de la cepa LaSota y de dos clones infecciosos identificados como NDFL y NDFLtag. NDFL es un clon infeccioso de una cepa lentogénica del virus de la enfermedad de Newcastle (LaSota E13-1) y NDFLtag es un clon infeccioso con un sitio de corte de la proteína de fusión mutado a un motivo virulento. NDFL y NDFLtag fueron descritos por Peeters y colaboradores. Los virus se inocularon en la conjuntiva ocular en grupos de pollos leghorn blancos y fueron comparados en un estudio de patogénesis para determinar inducción de enfermedad (signos clínicos, lesiones macroscópicas, histología, aislamiento viral y serología) y distribución viral (presencia de nucleoproteína viral y ARN mensajero detectados mediante inmunohistoquímica e hibridación *in situ*, respectivamente). A la evaluación de su patogénesis, la modificación en el sitio de corte de la proteína de fusión hacia un motivo virulento en el clon infeccioso solo incrementó ligeramente la gravedad de la enfermedad y la distribución viral, aunque mediante pruebas estándar de índice de patogenicidad se demostró un incremento dramático en la patogenicidad del clon NDFLtag. El hecho de que la mutación en el sitio de corte de la proteína de fusión del virus de la enfermedad de Newcastle NDFLtag tenga solo una pequeña influencia en la patogenia en pollos al compararse con los clones E13-1 o NDFL, sugiere que los efectos patogénicos del virus de la enfermedad de Newcastle no dependen exclusivamente del sitio de corte de la proteína de fusión.

Key words: Avian paramyxovirus 1, fusion protein cleavage site, immunohistochemistry, infectious clones, *in situ* hybridization, Newcastle disease, pathogenesis, reverse genetics

Abbreviations: BHI = brain-heart infusion broth; dpi = days postinoculation; EID_{50} = 50% embryo infectious dose; GB = Gilbert Boney; HA = hemagglutination; HI = hemagglutination inhibition; ICPI = intracerebral pathogenicity index; IHC = immunohistochemistry; ISH = *in situ* hybridization; IVPI = intravenous pathogenicity index; MDT = mean death time; mRNA = messenger RNA; ND = Newcastle disease; NDV = Newcastle disease virus; OIE = Office International des Epizooties; PBS = phosphate-buffered saline; PPMV-1 = paramyxovirus 1; RT-PCR = reverse transcription–polymerase chain reaction; SEPRL = Southeast Poultry Research Laboratory; SPF = specific pathogen free

Newcastle disease virus (NDV) varies widely in the type and severity of the disease it produces. Consequently, there are often difficulties in recognizing the clinical problem as Newcastle disease (ND) because of the variety of clinical signs and absence of diagnostic lesions (1,3,4). Newcastle disease virus is synonymous with avian paramyxovirus 1 in the order *Mononegavirales*, family *Paramyxoviridae*, subfamily *Paramyxovirinae*, genus *Avulavirus* (16,17). The virus strains are classified into highly virulent (velogenic) NDV, moderately virulent (mesogenic) NDV, and low virulent (lentogenic) NDV by standard pathogenicity index tests, such as the mean death time (MDT) in embryonating eggs, the intracerebral pathogenicity index (ICPI) in 1-day-old chicks, and the intravenous pathogenicity index (IVPI) in 6-wk-old chickens (2,4).

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In the United States, the intracloacal inoculation test is also used to differentiate velogenic pathotypes into viscerotropic or neurotropic strains (4).

The marked strain-dependent differences in tropism and virulence observed with NDV have been shown to depend upon the presence of cellular proteases required for cleavage site activation of the viral fusion glycoprotein precursor (9,10,18,19,26). Virulent (mesogenic and velogenic) strains have the motif ^{112}R/K-R-Q-R/K-R-F^{117}, allowing cleavage by proteases ubiquitously present in cells throughout the body, resulting in pantropic or systemic infection. In contrast, low virulent strains have sequences in the same region of ^{112}G/E-K/R-Q-G/E-R-L^{117} and require trypsin-like enzyme(s) for cleavage. Low virulent viruses, therefore, appear to be restricted to replication primarily in the respiratory and intestinal tracts, where endogenous trypsin-like enzymes naturally exist (4,10,18).

In this study, a wild-type low virulent virus (LaSota E13-1) and its infectious clones constructed by Peeters *et al.* (22), specifically, a low virulent infectious clone (NDFL) and the infectious clone with F cleavage site sequence mutated to the virulent motif (NDFLtag), were used, and the pathogenesis of the viruses was compared. For this pathogenesis assessment, groups of 4-wk-old specific-pathogen-free (SPF) chickens were inoculated with each virus, and the disease causation of the viruses was compared through clinical signs of disease and gross and histologic lesions. Viral distribution and replication in tissues were documented by immunohistochemistry (IHC) and *in situ* hybridization (ISH), respectively. In addition, pathogenicity indices were calculated using the standard pathogenicity index tests.

MATERIALS AND METHODS

Viruses. Two infectious clones constructed and provided by Peeters *et al.* (22) and their parent wild-type virus were used. These were 1) LaSota E13-1, virus from the third round of plaque purification on chicken embryo fibroblast cells of lentogenic NDV strain LaSota (ATCC VR-699); 2) NDFL, an infectious clone generated from cloned full-length complementary DNA of LaSota E13-1; 3) NDFLtag, NDFL with genetic modification of the fusion cleavage site. The cleavage site of NDFL (^{111}GGRQGRL^{117}) was converted to the consensus protease cleavage site of virulent NDV strains (^{111}GRRQRRF^{117}). Peeters *et al.* (22) classified NDFL as a lentogenic strain (ICPI = 0.00), whereas NDFLtag was classified as a mesogenic strain (ICPI = 1.28). Viruses were propagated in embryonating eggs at Southeast Poultry Research Laboratory (SEPRL), and the amnioallantoic fluid harvested from those infected eggs was used as inoculum after proper dilution.

Eggs and chickens. The source of embryonating chicken eggs and chickens was the SEPRL SPF white leghorn flock. Birds were housed in negative-pressure isolators under biosafety level (BSL)-3 agriculture conditions at SEPRL and provided feed and water *ad libitum.* Embryonating eggs were inoculated for viral propagation, isolation, titration, and the mean death time (MDT) test. Chickens were inoculated for the pathogenesis study, the ICPI tests, the IVPI test, and the intracloacal inoculation tests.

Pathogenesis assessment in chickens. Four groups of ten 4-wk-old SPF white leghorn chickens were inoculated *via* instillation bilaterally in the conjunctival sac with 0.1 ml of inoculum of wild-type virus (LaSota E13-1), its clone (NDFL), or the clone with fusion gene modification (NDFLtag), as well as phosphate-buffered saline (PBS) as a noninfected control. The target dose of inoculum was $10^{5.0}$ 50% embryo infectious doses (EID_{50}). The actual infectious doses as determined by titration in chicken embryonating eggs were $10^{4.7}$ EID_{50}/0.1 ml of LaSota E13-1, $10^{3.9}$ EID_{50}/0.1 ml of NDFL, $10^{4.9}$ EID_{50}/0.1 ml of NDFLtag. The birds were monitored clinically every day, and two birds of each group were euthanatized after taking oropharyngeal and cloacal swabs at 2, 5, 10, and 14 days postinoculation (dpi). Tissues (eyelid, spleen, thymus, bursa, Harderian gland, proventriculus, small intestine, cecal tonsils, large intestine, air sac, trachea, lung, heart, esophagus, pharynx, crop, brain, liver, pancreas, kidney, comb, head of left femur including bone marrow, and turbinate) were collected, and all sampled tissues were fixed by immersion in 10% neutral buffered formalin for approximately 52 hr. Blood samples for serology were also collected at 10 and 14 dpi. The sections of femur and turbinates were decalcified in 5% formic acid for 3–4 hr. All sampled tissues were routinely processed into paraffin, and 3-μm sections were cut for hematoxylin and eosin staining, IHC, and ISH.

Immunohistochemistry. All sampled tissues were examined by IHC with the following protocol to detect viral nucleoprotein. After deparaffinization, tissue sections were subjected to antigen retrieval by microwaving for 10 min at full power in Vector antigen unmasking solution (Vector Laboratories, Burlingame, CA) followed by blocking with universal blocking reagent (Biogenex, San Ramon, CA) as recommended by the manufacturer. The primary antibody, made in rabbit, was anti-peptide (nucleoprotein), used at 1:8000 dilution (13), and the secondary antibody was biotinylated goat anti-rabbit antibody (Vector Laboratories). The detection system was avidin–biotin–alkaline phosphatase (Vector Laboratories), and the substrate was Vector Red (Vector Laboratories). Sections were counterstained lightly with hematoxylin and coverslipped with Permount for a permanent record.

***In situ* hybridization.** Selected tissue sections were probed with a negative sense digoxigenin-labeled 850-base riboprobe representing the 5′ end of the matrix gene of B1. Protocol was as described previously (5,13,14,15). Briefly, tissue sections were deparaffinized, rehydrated, and digested with 30 μg/ml proteinase K for 15 min at 37 C. Hybridization was conducted overnight at 42 C with approximately 20 ng of probe in prehybridization solution. After stringent washes, antidigoxigenin alkaline phosphatase was added to the sections. The development was with chromogen/substrate nitroblue tetrazolium/5-bromo, 4-chloro, 3-indolylphosphate. Tissues were counterstained lightly with hematoxylin and coverslipped.

Virus isolation and titration of swabs. For those birds used in the pathogenesis assessment experiment, immediately prior to euthanasia, oral and cloacal swabs were obtained from each bird and placed in separate tubes containing 1.5 ml of brain-heart infusion broth (BHI) with antibiotics (2000 unit penicillin G/ml BHI, 200 μg gentamicin sulfate/ml BHI, and 4 μg amphotericin B/ml BHI; Sigma Chemical Co., St. Louis, MO). Swab sample tubes were centrifuged at 1000 × *g* for 20 min, and the supernatant was removed for virus isolation and titration. Virus infectivity titers were calculated from the results of inoculation of 9- to 10-day-old SPF embryonating chicken eggs with serial 10-fold dilution in BHI containing antibiotics (100 unit penicillin G/ml BHI and 50 μg gentamicin sulfate/ml BHI). NDV-infected dead or surviving embryos were identified by hemagglutination (HA) activity in amnioallantoic fluid harvested from chilled eggs. NDV was confirmed in HA-positive samples by hemagglutination-inhibition (HI) test with NDV-specific antiserum.

Serology. The HA and HI tests were conducted by conventional microtiter methods (11) with serum separated from the blood samples taken at 10 and 14 dpi. Four HA units of beta propiolactone inactivated NDV LaSota was used as test antigen in completing the HI tests.

Nucleotide and predicted amino acid sequence analysis. Newcastle disease virus isolated from oral swabs of birds at 4 dpi was replicated in embryonating eggs, and RNA was extracted directly from amnioallantoic fluid. Oligonucleotide reverse transcription–polymerase chain reaction (RT-PCR) primers were designed to amplify regions of the fusion protein gene, including the fusion protein cleavage site and the matrix protein gene region encoding the nuclear localization signal of the matrix protein. The degenerate oligonucleotide primer pairs were 5′-TCGAGICTGTACAATCTTGC-3′ (sense) and 5′-CTGCCACT-GCTAGTTGIGATAATCC-3′ (antisense) (23). A single tube reverse RT-PCR for genomic NDV RNA was completed with Superscript™ (Life Technologies, Gaithersburg, MD) and Amplitaq™ (Applied Biosystems, Inc., Foster City, CA) polymerase. Amplification products were separated by gel electrophoresis in 1.0% agarose with Tris-borate buffer, stained with ethidium bromide. Amplification products were purified with Microcon™ (Amicon, Belford, MA) spin filters and

spectrophotometrically quantified. Sequentially, amplification products were cloned with the TA cloning system™ according to the methods described by the manufacturer (Invitrogen, San Diego, CA). Direct double-stranded nucleotide sequencing was completed with *Taq* polymerase (Applied Biosystems, Inc.) with the oligonucleotide primers used for RT-PCR, fluorescent-labeled dideoxynucleotides, and an automated nucleic acid sequencer.

Pathogenicity index tests. Virus virulence was determined with standard pathogenicity tests that were performed and interpreted as described (2,13,21). Briefly, those tests were completed as follows. Embryos inoculated with serial dilutions were candled twice daily to determine time of embryo death. The mean death time (MDT) for each virus was calculated as the mean time of embryo death in the highest dilution that had 100% deaths. The inoculum for the ICPI in 1-day-old chicks, the IVPI in ten 6-wk-old chickens, and the intracloacal inoculation test in five 6-wk-old chickens was 0.1 ml of a 1/10 dilution of infective allantoic fluid, an approximately 1000-fold higher infectivity titer dosage than the inoculum in the pathogenesis assessment. Birds were scored as normal, sick or paralyzed, or dead daily to compile a score for an 8-day observation in the ICPI and a 10-day observation in the IVPI and intracloacal test.

RESULTS

Pathogenesis assessment in chickens—clinical disease, gross pathology, histopathology, IHC, ISH, virus isolation, and serology. Clinical signs were not overt in any birds inoculated with LaSota E13-1 or NDFL. One bird infected with NDFLtag showed nasal discharge at 5 dpi. In birds infected with LaSota E13-1, slightly enlarged spleens were observed at 2, 10, and 14 dpi. Slight thymic enlargement was present in the birds infected with LaSota E13-1 at 14 dpi and with NDFLtag at 5 dpi. The only overt histologic changes were detected in eyelids, where, among infected birds, there was infiltration of large numbers of lymphocytes, often extending into the mucosa, submucosa, and muscularis. This pattern was consistent with all three viruses (NDFL, Fig. 1; for comparison, PBS, Fig. 2) but was most extensive in those infected with NDFLtag. Immunohistochemistry revealed NDV nucleoprotein in a few nasal glandular epithelial cells at 2 dpi in a bird infected with LaSota E13-1 (Fig. 3). Birds infected with NDFLtag had viral nucleoprotein in the mucosal epithelial cells and in infiltrating lymphocytes in the eyelids at 2 and 5 dpi and in pharyngeal mucosa at 10 dpi. Only eyelids from the bird with NDFLtag at 2 dpi showed presence of large amounts of viral nucleoprotein (Fig. 4), whereas others had minimal staining. Low levels of viral messenger RNA (mRNA) were detected by ISH in lymphocytes infiltrating into eyelids of a bird infected with LaSota E13-1 at 10 dpi (Fig. 5) and in the salivary glandular epithelial cells in a bird infected with NDFLtag at 10 dpi (Fig. 6).

Nucleotide and predicted amino acid sequence analysis. LaSota E13-1 and NDFL have the ^{109}SGGGRQGRLIG^{119} sequence, whereas NDFLtag has the sequence ^{109}SGGRRQRRFIG^{119} containing the diagnostic pair of dibasic amino acids (RRQRR) associated with the primary molecular determinant of virulence (9,22). The results of sequence analysis demonstrated that no change occurred within the fusion cleavage site after viral propagation in embryonating eggs and a passage in 4-wk-old chickens for the pathogenesis study.

Virus isolation, titration of swabs, and serology. Results are presented in Table 1. Virus was isolated from oral swabs from birds infected with LaSota E13-1, NDFL, and NDFLtag at 2, 5, and 10 dpi; at 2 and 5 dpi; and at 2 and 5 dpi, respectively. From cloacal swabs, virus was isolated only once, from a bird infected with NDFLtag at 10 dpi. No virus was isolated from noninfected birds. Results of serologic testing are presented in Table 2. All blood samples from infected birds, except a bird with LaSota E13-1 at 10 dpi, had increased HI titers. Blood from birds infected with NDFLtag had the highest HI titers among the three viruses at both time points.

MDTs, ICPIs, IVPIs, and intracloacal pathogenicity test. Results are summarized in Table 3. There was very little difference between LaSota E13-1 and NDFL in the MDT, ICPI, or IVPI. In contrast, NDFLtag had decreased MDT and increased ICPI and IVPI, which are consistent with higher pathogenicity of NDFLtag compared to LaSota E13-1 and NDFL. In addition, intracloacal inoculation test was performed with NDFLtag, and two of five birds showed leg paralysis at 8 dpi. These were euthanized at 9 dpi due to severe clinical signs.

DISCUSSION

The fusion protein cleavage amino acid sequence has been postulated to be a primary determinant of NDV virulence (9,20), and identification of the multiple basic amino acids and phenylalanine at that site in an isolate is one of the criteria now accepted by the World Organization for Animal Health (formerly known as the Office International des Epizooties and still recognized by the designated abbreviation, OIE) to characterize the isolate as virulent and the cause of Newcastle disease (21). However, strains with identical or similar virulent basic amino acid sequence at the fusion cleavage site may produce a wide range of clinical disease manifestations in chickens. For example, the Turkey ND neurotropic velogenic virus shares the same fusion protein cleavage site sequence as the mesogenic Anhinga virus, and the mesogenic Roakin virus has the same fusion protein cleavage site as the neurotropic velogenic Texas Gilbert-Boney virus (23,24); however, these viruses all behave quite differently clinically as reported by Brown *et al.* (5) in prior pathogenesis studies. In those studies severe clinical disease and mortality was only associated with the velogenic viruses. The variant NDV strains, pigeon paramyxovirus 1 (PPMV-1), with typical virulent fusion cleavage site were usually of moderate virulence by pathogenicity index assays, but an occasional virus is of low virulence (6). Some depression and nervous signs with only occasional mortality was reported in pathogenesis studies of PPMV-1 isolates with ICPIs similar to NDFLtag (13). Therefore, it is apparent that even though NDV isolates share the common feature of a virulent fusion cleavage site, there must be other contributory factors that determine tissue tropism and extent of clinical disease.

In this study, the role of fusion cleavage activation site gene mutations on the pathogenesis of NDV in chickens was investigated. A wild-type lentogenic virus (LaSota E13-1), its infectious clone (NDFL), and the infectious clone with modification of the low virulent fusion cleavage site into the consensus sequence of the virulent strain (NDFLtag) described previously by Peeters *et al.* (22) were used for the study. The viruses were compared by pathogenesis assessment with 4-wk-old chickens, using a natural route of inoculation (intraconjunctivally) and following clinical disease and pathologic features, including viral distribution to tissues. LaSota E13-1 and NDFL acted very similarly. NDFLtag caused slightly more severe disease. Only one bird infected with NDFLtag showed mild nasal discharge, and there were no other clinical signs of disease. Histologically, there were marked lymphocytic infiltrates in the eyelids of birds infected with NDFLtag, while such infiltrates were relatively less extensive in birds infected with either LaSota E13-1 or NDFL. Distribution of viral nucleoprotein and mRNA was slightly more widespread in birds infected with NDFLtag than with either LaSota E13-1 or NDFL. With LaSota E13-1 and NDFL, viral distribution and replication were limited to inoculation sites;

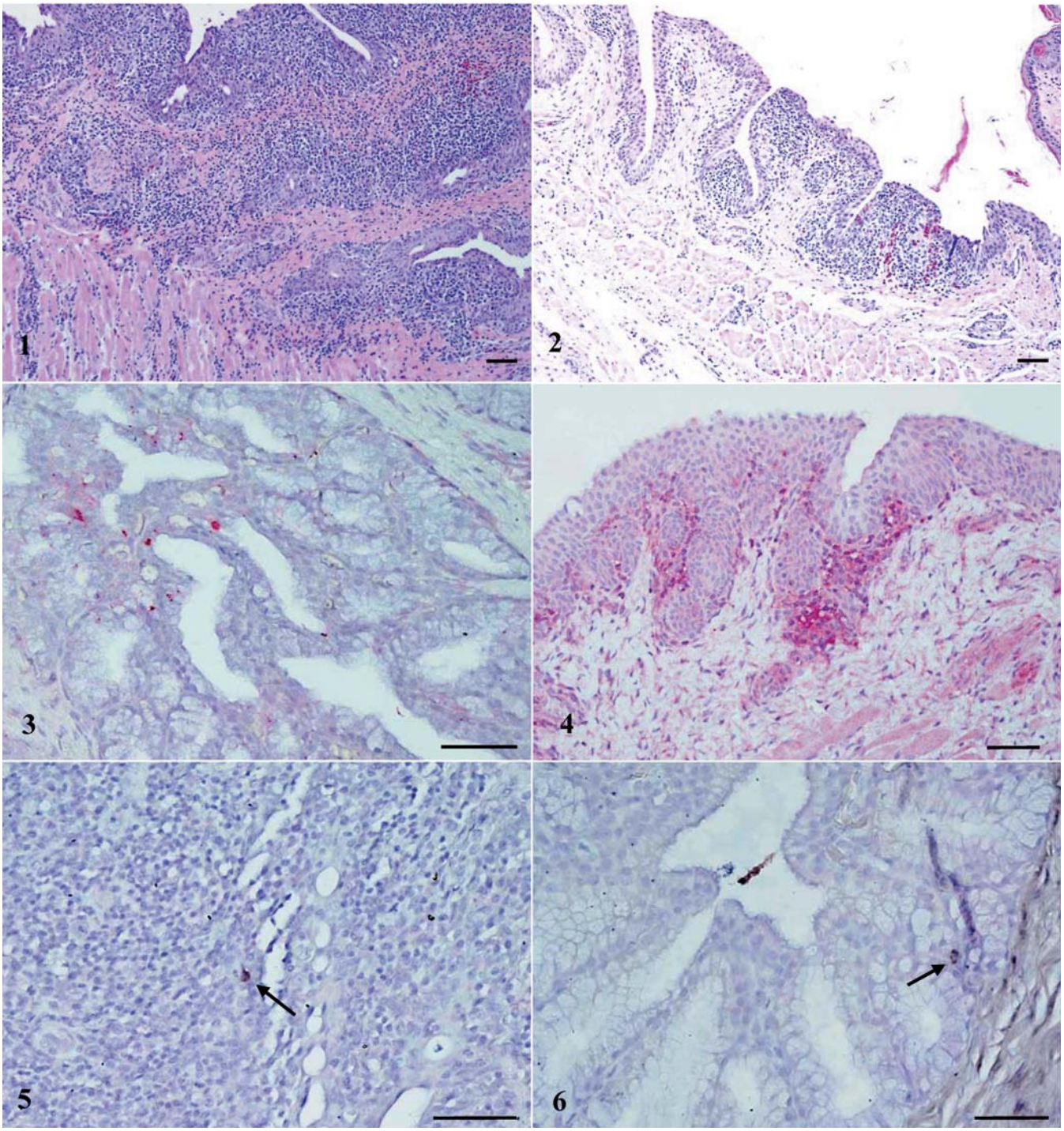

Fig. 1. Eyelid; chicken infected 5 days previously with NDFL. Extensive lymphocytic infiltrates into the mucosa, submucosa, and muscularis. Hematoxylin and eosin stain. Bar = 30 μm.

Fig. 2. Eyelid; noninfected chicken. Lymphocellular aggregates in the mucosa and superficial submucosa. Hematoxylin and eosin stain. Bar = 30 μm.

Fig. 3. Nasal gland; chicken infected 2 days previously with LaSota E13-1. Viral antigen was detected in a few glandular epithelial cells. Immunohistochemistry for NDV nucleoprotein, Mayer's hematoxylin counterstain. Bar = 30 μm.

Fig. 4. Eyelid; chicken infected 2 days previously with NDFLtag. Viral antigen was detected in mucosal epithelial cells and infiltrating lymphocytes and macrophages in the mucosa and submucosa. Immunohistochemistry for NDV nucleoprotein, Mayer's hematoxylin counterstain. Bar = 30 μm.

Fig. 5. Eyelid; chicken infected 10 days previously with LaSota E13-1. Viral nucleic acid was detected in a lymphocyte in the submucosa (arrow). *In situ* hybridization for NDV mRNA, Mayer's hematoxylin counterstain. Bar = 30 μm.

Fig. 6. Salivary gland; chicken infected 10 days previously with NDFLtag. Viral nucleic acid was detected in a glandular epithelial cell (arrow). *In situ* hybridization for NDV mRNA, Mayer's hematoxylin counterstain. Bar = 30 μm.

Table 1. Virus isolation and titration of oral and cloacal swab samples from 4-wk-old chickens infected 2, 5, 10, and 14 days previously with LaSota E13-1, NDFL, and NDFLtag.

		PBS		LaSota E13-1 ($10^{4.7}$)[A]		NDFL ($10^{3.9}$)[A]		NDFLtag ($10^{4.9}$)[A]	
dpi[B]	ID[C]	Oral	Cloacal	Oral	Cloacal	Oral	Cloacal	Oral	Cloacal
2	a	—[D]	—	$10^{2.2}$,[E]	—	$10^{2.9}$	—	$10^{3.9}$	—
	b	—	—	$10^{0.1}$	—	$10^{2.9}$	—	$10^{1.5}$	—
5	c	—	—	$10^{3.2}$	—	$10^{3.2}$	—	$10^{3.2}$	—
	d	—	—	$10^{\geq 4.2}$	—	$10^{\geq 4.2}$	—	$10^{3.2}$	—
10	e	—	—	$10^{0.3}$	—	—	—	—	—
	f	—	—	$10^{2.7}$	—	—	—	—	$10^{0.3}$
14	g	—	—	—	—	—	—	—	—
	h	—	—	—	—	—	—	—	—

[A]Number in parentheses is the viral titer of inoculum per 0.1 ml.
[B]Days postinoculation.
[C]Bird identification.
[D]No virus recovered from undiluted swab fluids inoculated into three eggs, 0.2 ml per egg.
[E]Viral titer of swab sample per 0.1 ml.

however, NDFLtag was distributed to and replicated in the epithelial cells of the salivary glands and the pharynx in addition to the inoculation site. Extensive replication at the site of inoculation might have been what allowed the NDFLtag to extend to salivary glands and pharynx. Virus isolation and serology assays also revealed differences among the viruses. A bird infected with NDFLtag was the only one that shed virus from the cloaca. Serologically, the HI titers of birds infected with NDFLtag were higher than those of birds with LaSota E13-1 and NDFL. These results suggest that NDFLtag replicated within the birds to a greater degree than did LaSota E13-1 and NDFL. It has been reported that selected fusion cleavage site mutants of NDFL give rise to revertants with a velogenic cleavage site by point mutations after just one passage in chicken brains (7). In our study, sequence analysis of virus isolated from swabs demonstrated that the fusion cleavage site of each of the viruses was unchanged after chicken passage.

The standard pathogenicity index tests were performed to characterize the viruses that were used for the pathogenesis study. The present ICPI values for NDFL (0.08) and NDFLtag (1.60) and IVPI values of NDFLtag (1.71) were higher than those previously reported by Peeters *et al.* (NDFL ICPI = 0.00, NDFLtag ICPI = 1.28) (22) and by de Leeuw *et al.* (NDFLtag IVPI = 0.79) (8). Although the same pathogenicity test protocols were reportedly followed in this and prior studies, slightly different experimental conditions among the laboratories, such as chicken breed (12) and age (25), could have influenced the index scores. Even though there were differences between the pathogenicity test results of the present and prior studies, those differences did not change the classification of pathotype based on the accepted standards (2). The isolate E13-1 and its infectious clone NDFL are lentogens, and the lower MDT and higher ICPI and IVPI values observed with NDFLtag are typical of viruses classified as mesogens. The mutation of the fusion cleavage site of NDFL to the virulent motif in NDFLtag did not increase the pathogenicity test results to a level typical for a velogenic virus, a MDT $\leq$ 60 hr, an ICPI $\geq$ 1.5, and an IVPI $\geq$ 2.0 (2).

The apparent capacity of NDFLtag to establish a systemic infection and to cause histologic damages in 4-wk-old SPF chickens was markedly decreased compared to naturally virulent NDV isolates with a similar virulent fusion cleavage site. Systemic viral distribution and severe tissue damage, especially in lymphoid tissues, were seen in chickens infected with viscerotropic velogenic NDV isolates (e.g., California 1083), which caused early onset of severe clinical signs and high mortality (5). Neurotropic velogenic viruses (e.g., Texas GB and Turkey ND) caused less severe disease than did the viscerotropic viruses, and viral mRNA was detected in multiple organs, especially in the brain (5). Mesogenic isolates (e.g., Roakin and Anhinga), the same pathotype as NDFLtag, did not cause any overt clinical disease (5, 14), but viral nucleoprotein (14) and mRNA (5) were detected in the cardiac myofibers as well as the inoculation sites (5,14). Encephalitis and neuronal degeneration were also reported in birds inoculated with mesogenic Anhinga strain (ICPI = 1.31) (14). Although naturally virulent viruses, particularly the velogens, do disseminate and replicate in

Table 2. Newcastle disease virus hemagglutinin-inhibition (HI) titers of serum collected from 4-wk-old chickens at day 0 and infected 10 and 14 days previously with LaSota E13-1, NDFL, and NDFLtag.

dpi[A]	ID[B]	PBS	LaSota E13-1	NDFL	NDFLtag
0		<2[C]	<2	<2	<2
10	e	<2	16	8	64
	f	<2	<2	16	32
14	g	<2	8	16	64
	h	<2	64	64	128

[A]Days postinoculation.
[B]Bird identification.
[C]HI titer.

Table 3. Pathogenicity index tests and amino acid sequence at the fusion cleavage site of LaSota E13-1, NDFL, and NDFLtag.

Test	LaSota E13-1	NDFL	NDFLtag
MDT[A]	125	123	77
ICPI[B]	0.01	0.08	1.60
IVPI[C]	0.01	0.00	1.71
Intracloacal[D]	ND[E]	ND	2 of 5 dead[F]
F cleavage site[G]	Low virulent	Low virulent	Virulent

[A]Mean death time in embryonating eggs.
[B]Intracerebral pathogenicity index.
[C]Intravenous pathogenicity index.
[D]Intracloacal inoculation test.
[E]Not done.
[F]Two of total five birds were euthanatized at 9 dpi because of severe clinical signs.
[G]Fusion cleavage site sequence; low virulent = ^{111}GGRQGRLIG^{119}; virulent = ^{111}GRRQRRFIG^{119}.

multiple tissues, distribution of NDFLtag was apparently restricted to the site of inoculation and epithelial cells of salivary glands and pharynx.

The mutation in the fusion cleavage site to generate NDFLtag from NDFL increased, but only slightly, the virulence of the virus based upon extent of clinical disease, histologic damage, and viral distribution in 4-wk-old chickens compared to the parent virus. The histologic damage was less severe and viral distribution was less extensive than observed in prior studies with those naturally occurring mesogenic and velogenic viruses that also have a virulent fusion cleavage activation site. The mild influence on the pathogenesis of the fusion cleavage site mutation that generated NDFLtag suggests that virulence of NDV is not dependent on the fusion cleavage site alone.

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ACKNOWLEDGMENTS

We extend our gratitude to Phillip Curry and Robin Kuntz for excellent technical assistance. This work was supported by the U.S. Poultry and Egg Association (project number 368) and the USDA Agricultural Research Service (CRIS number 6612-32000-038-OOD).